

# Corticotropin-Releasing Hormone Directly Stimulates Thermogenesis in Skeletal Muscle Possibly through Substrate Cycling between *de Novo* Lipogenesis and Lipid Oxidation

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The mechanisms by which CRH and related peptides (*i.e.* the CRH/urocortin system) exert their control over thermogenesis and weight regulation have until now focused only upon their effects on brain centers controlling sympathetic outflow. Using a method that involves repeated oxygen uptake determinations in intact mouse skeletal muscle, we report here that CRH can act directly on skeletal muscle to stimulate thermogenesis, an effect that is more pronounced in oxidative than in glycolytic muscles and that can be inhibited by a selective CRH-R2 antagonist or blunted by a nonselective CRH receptor antagonist. This thermogenic effect of CRH can also be blocked by interference along pathways of *de novo* lipogenesis and lipid oxidation, as well as by inhibitors of phosphatidylinositol 3-kinase or AMP-activated protein kinase.

Taken together, these studies demonstrate that CRH can directly stimulate thermogenesis in skeletal muscle, and in addition raise the possibility that this thermogenic effect, which requires both phosphatidylinositol 3-kinase and AMP-activated protein kinase signaling, might occur via substrate cycling between *de novo* lipogenesis and lipid oxidation. The effect of CRH in directly stimulating thermogenesis in skeletal muscle underscores a potentially important peripheral role for the CRH/urocortin system in the control of thermogenesis in this tissue, in its protection against excessive intramyocellular lipid storage, and hence against skeletal muscle lipotoxicity and insulin resistance. (*Endocrinology* 147: 31-38, 2006)

THE CRH SYSTEM consists of distinct populations of CRH-expressing neurons, a CRH-binding protein, a family of receptors encoded by two different genes (*crhr-1* and *crhr-2*) with several splice variants, and several related peptides (urocortin, urocortin 2, and urocortin 3), which are also ligands for CRH receptors. These components of the CRH system constitute an elaborate network of neuronal pathways that play a pivotal role in orchestrating the body's overall response to stress and to stress-induced activation of the hypothalamic-pituitary-adrenal axis (1). The CRH system also interacts with brain circuitries controlling food intake and thermogenesis (2). Its role in weight regulation has been linked to its interaction with peripheral hormonal signals that inform the brain about the status of the body's fat reserves (2). In particular, the importance of the CRH system as a target for the anorectic and thermogenic effects of the adipocyte-secreted hormone leptin has been emphasized in

studies demonstrating that 1) leptin down-regulates the expression of hypothalamic CRH (3), thereby reducing the hypothalamic pituitary-adrenal axis activity, and 2) when administered centrally, CRH antagonists prevent the anorectic effects of leptin (4), whereas CRH, like leptin (5-7), stimulates whole-body oxygen consumption (8) and sympathetically mediated thermogenesis in brown adipose tissue (9, 10).

Leptin also exerts direct peripheral effects on skeletal muscle metabolism, namely by stimulating glucose use in a phosphatidylinositol 3-kinase (PI3K)-dependent manner (11) or fatty acid oxidation through activation of the AMP-activated protein kinase (AMPK) and ACC [acetyl coenzyme A (CoA) carboxylase] axis (12). More recently, leptin has also been shown to have direct effects on skeletal muscle thermogenesis (13), through mechanisms that involve substrate cycling between *de novo* lipogenesis and lipid oxidation (14, 15). Such an energy-dissipating pathway might not only contribute to the stimulatory effect of leptin replacement on energy expenditure after weight loss (16, 17) but may also be the mechanism by which leptin regulates intramyocellular lipid stores and protects skeletal muscle against lipid-induced stress (lipotoxicity) (18). Whether, as a circulating hormone, CRH might play similar roles at the periphery is not known. We therefore decided to test the hypothesis that CRH, like leptin, may also directly stimulate thermogenesis in skeletal

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Abbreviations: ACC, Acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; araA, 9- $\beta$ -D-arabinofuranoside; CoA, coenzyme A; CPT, carnitine palmitoyl transferase-1; EDL, extensor digitorum longus; IRS, insulin receptor substrate;  $\text{MO}_2$ , respiratory rate; PI3K, phosphatidylinositol 3-kinase; PY, phosphotyrosine; UCP, uncoupling protein.

muscle on the basis of the following: 1) a splice variant of the CRH receptor encoded by the *crhr-2* gene (CRH-R2 $\beta$ ) is also expressed in skeletal muscle (19, 20), and 2) peripheral infusion of CRH stimulates thermogenesis and fat oxidation in humans without signs of increased adipose tissue lipolysis or activation of the sympatho-adrenal system (21).

To this end, we have evaluated the direct effect of CRH on skeletal muscle thermogenesis by assessing changes in the respiration rate of intact murine skeletal muscle (*ex vivo*) by a method that involves repeated oxygen uptake determinations for several hours in indirect microcalorimeter chambers. We subsequently examined the extent to which the thermogenic response to CRH might be altered by selective inhibitors of key control points in pathways of *de novo* lipogenesis and lipid oxidation, as previously reported for leptin (14). In addition, we also investigated the role of PI3K and AMPK signaling in the peripheral thermogenic effect of CRH in skeletal muscle.

## Materials and Methods

### Mice

Intact muscles were obtained from 7- to 8-wk-old male BALB/cByJlco mice (Charles River Laboratories, L'Arbresle, France). Before the start of each experiment, the mice were adapted for at least 1 wk to a temperature-controlled room (22°C) with a 12-h light, 12-h dark cycle. They had free access to tap water and were maintained on a commercial pelleted laboratory diet (Provimi-Lacta, Cossonay, Switzerland) consisting, by energy, of 24% protein, 66% carbohydrates, and 10% fat. All animals used in the present studies were maintained in accordance with our institute's regulations and guide for the care and use of laboratory animals.

### Muscle tissue preparations

For *ex vivo* calorimetric measurements, soleus and/or extensor digitorum longus (EDL) muscles were carefully dissected out intact together with their tendons and freed of loosely attached connective tissue. They were then placed on a stainless steel frame, at physiological resting length, in the test chambers of a twin indirect microcalorimeters perfused with Krebs-Ringer bicarbonate buffer at 30°C, as described previously (13).

### Measurement of tissue respiration rate

The respiratory rate (MO<sub>2</sub>) of skeletal muscle was measured for about 7 h by a method involving repeated O<sub>2</sub> uptake determinations in our twin microcalorimeter chambers as described by Barde *et al.* (22). The O<sub>2</sub> partial pressure of a bubble-free liquid phase enclosed in a thick-walled Lucite chamber was measured by a Clark O<sub>2</sub> electrode connected to a polarographic circuit, whose output voltage is directly proportional to O<sub>2</sub> partial pressure. At about 10-min intervals, a peristaltic pump partially exchanged the solution for a fresh one within 2–3 min. All values for MO<sub>2</sub> were taken during steady-state respiration. The basal steady-state MO<sub>2</sub> was taken as the average value between 120 and 150 min after placing the muscle preparations in the experimental chambers. For each hormone, receptor antagonist or inhibitor that was subsequently added, the steady-state respiration value was taken as the average value between 90 and 120 min after its administration. The choice of an effective concentration for CRH antagonists or inhibitors was based upon preliminary calorimetric experiments that involved testing several concentrations of each antagonist or inhibitor on muscle MO<sub>2</sub>. Because of the long duration (2–2.5 h) necessary for assessing each steady-state MO<sub>2</sub> value, only three steady-state MO<sub>2</sub> values, *i.e.* the basal MO<sub>2</sub> value and sequential effects of only two concentrations of antagonists or inhibitors—could be monitored in any given intact skeletal muscle on a given day. The selected concentration for CRH receptor antagonists corresponded to the lowest concentration tested that was effective in inhibiting CRH's stimulatory effect on MO<sub>2</sub>, but which, in its own rights, did

not have an inhibitory effect on basal MO<sub>2</sub>; this corresponded to 1 µg/ml for each of the two CRH receptor antagonists used: antisauvagine-30 and astressin. Furthermore, in testing whether CRH stimulates thermogenesis by the same mechanism (substrate cycling) that we previously proposed for leptin (14), we used the same concentration of the various inhibitors that we previously found to completely block the direct effect of leptin on muscle thermogenesis (13, 14). We believe that these concentrations are close to the minimal effective dose in our assay because concentrations corresponding to five times higher dilution of each inhibitor failed to produce statistically significant effects on CRH-induced increases in muscle MO<sub>2</sub>.

### AMPK/ACC phosphorylation and PI3K/Akt assays

Soleus muscles were incubated *ex vivo* in Krebs-Ringer buffer alone for 30 min, followed by incubation alone or in presence of a test hormone or drug for 15 min, and then immediately frozen in liquid nitrogen. Frozen muscles were homogenized and incubated in lysis buffer [20 mM Tris-HCl, 138 mM NaCl, 2.7 mM KCl, 5% (vol/vol) glycerol, Nonidet P-40 and protease/phosphatase inhibitors] for 15 min. After centrifugation at 13,000 rpm for 15 min, protein concentration was quantified and the protein extracts were used for measurement of AMPK Thr172, ACC Ser79, and protein kinase B/Akt (referred to as Akt) Ser473 phosphorylation, or PI3K activity. For AMPK phosphorylation, 200 µg of protein extract were immunoprecipitated with phospho-AMPK $\alpha$  (Thr172) or phospho-ACC (Ser 79) polyclonal antibodies (Cell Signaling, Beverly, MA). The samples were then separated on a 10% SDS-PAGE gel and blotted on a polyvinylidene difluoride membrane that was analyzed with same phospho-AMPK $\alpha$  (Thr172) or phospho-ACC (Ser 79) polyclonal antibodies. For PI3K assay, 200 µg of protein extract were immunoprecipitated either with phosphotyrosine (PY) monoclonal antibody (Cell Signaling), with antipan p85 rabbit polyclonal antibody (obtained from M. P. Wymann's laboratory), insulin receptor substrate (IRS) 1 (Cell Signaling) or IRS2 (Upstate Biotechnology, Lake Placid, NY) polyclonal antibody. The kinase reaction, thin-layer chromatography separation, and signal detection were performed as previously described by Pirola *et al.* (23). For Akt phosphorylation, the protein extract was analyzed by immunoblot using Akt P-Ser 473-specific antibody (Cell Signaling), the membrane was then stripped from the Akt P-Ser 473-specific antibody and treated with Akt antibodies (Cell Signaling).

### Chemicals and drugs

All chemicals were purchased from Fluka (Buchs, Switzerland). Human CRH was synthesized by Bachem (Torrance, CA), and purity was confirmed by HPLC. The peptide, as the acetate salts, was reconstituted to a final concentration of 0.5 mg/ml (free base) in 0.9% saline acidified with glacial acetic acid. The peptides were aliquoted and frozen at –70°C until the day of use. Stresscopin-related peptide (human) or urocortin 2 was purchased from Bachem (Merseyside, UK), whereas Astressin and antisauvagine-30 were purchased from Polypeptide Laboratories Inc. (Torrance, CA), purity confirmed by HPLC, and the products supplied as acetate salts. Wortmannin and hydroxy-citrate from Calbiochem (Luzern, Switzerland), cerulenin from Fluka, and LY294002 as well as adenosine 9- $\beta$ -D-arabinofuranoside (araA) from Sigma (St. Louis, MO). Eto-moxir was a generous gift from Dr. W. Langhans (Zurich, Switzerland).

### Statistics

The analysis of data on changes in MO<sub>2</sub> in response to CRH and other drugs was performed either by paired *t* test or by ANOVA with repeated measures, using the statistical software STATISTIK (St. Paul, MN). Upon detection of significant increases by ANOVA, *post hoc* pairwise comparisons were conducted using Scheffé's test, with the level of statistical significance taken as *P* < 0.05.

## Results

### Direct effect of CRH on skeletal muscle thermogenesis

The addition of CRH, at 0.1 or 0.5 µg/ml, to the intact skeletal muscle preparations, resulted in significant increases in MO<sub>2</sub> above basal rate in the soleus muscle, namely by 20

and 25% ( $P < 0.01$ ), respectively (Fig. 1A); at higher concentrations (1  $\mu\text{g/ml}$ ), the addition of CRH did not lead to further increase in  $\text{MO}_2$  (data not shown). A stimulatory effect of CRH on  $\text{MO}_2$  is also observed in EDL muscles from these same mice, but this increase ( $\sim 7\%$  above basal values,  $P < 0.05$ ) is much less pronounced than in the soleus muscle (Soleus *vs.* EDL response,  $P < 0.01$ ). Real-time PCR data from our laboratory indicate that this muscle type difference in  $\text{MO}_2$  response cannot be attributed to differential gene expression of CRH-R2 $\beta$  because the mRNA levels were similar in both soleus and EDL muscles. Because the CRH effect on  $\text{MO}_2$  in EDL is small, subsequent studies were performed only in the soleus muscle whose response to CRH is quantitatively more important. To test whether the observed direct effect of CRH on muscle thermogenesis was due to binding of CRH to CRH-R2 $\beta$  receptors (the only CRH receptor isoform so far reported to be expressed in skeletal muscle), we investigated whether the CRH-induced increase in soleus muscle  $\text{MO}_2$  could be prevented by the presence of

antisauvagine-30, a selective CRH-R2 antagonist, and whether it could be mimicked by urocortin 2, a selective CRH-R2 agonist. The results, presented in Fig. 1, B and C, indicate that urocortin 2 also stimulates muscle  $\text{MO}_2$  (+14% above basal values,  $P < 0.001$ ) maximally at 0.25  $\mu\text{g/ml}$ ; at lower doses tested in preliminary studies (data not shown), urocortin 2 had either no effect relative to basal  $\text{MO}_2$  at 0.05  $\mu\text{g/ml}$  or increased muscle  $\text{MO}_2$  by about 10–12% ( $P < 0.05$ ) at 0.1  $\mu\text{g/ml}$ . Antisauvagine-30, at concentrations that do not have an effect on basal  $\text{MO}_2$  (Fig. 1D), resulted in a partial inhibition of the stimulatory effect of CRH or urocortin 2 on muscle  $\text{MO}_2$  ( $P < 0.05$ ), and its withdrawal resulted in an increase in muscle  $\text{MO}_2$  ( $P < 0.01$ ) in response to continuous administration of CRH or urocortin 2 (Fig. 1, B and C). However, complete blunting of the thermogenic effect of CRH and urocortin 2 can be observed in presence of the nonselective CRH receptor antagonist, astressin, at concentrations that do not have an effect on basal  $\text{MO}_2$  (Fig. 1D). Indeed, as also shown in Fig. 1, B and C, CRH (or urocortin 2) failed to

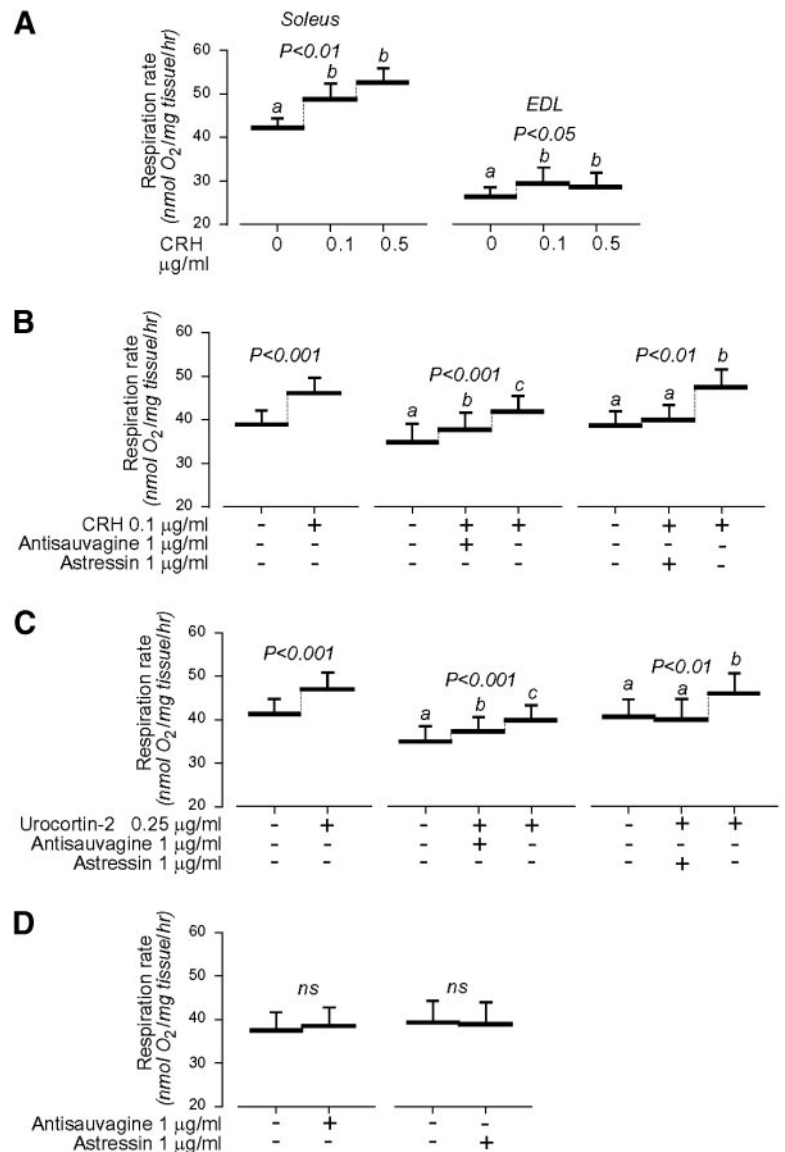


FIG. 1. Steady-state  $\text{MO}_2$  of: A, soleus and EDL muscles in the basal state and during sequential addition of 0.1 or 0.5  $\mu\text{g/ml}$  of CRH; B, soleus muscles in presence or absence of CRH, antisauvagine-30 (a selective CRH-R2 antagonist) or astressin (a nonselective CRH receptor antagonist); C, soleus muscles in presence or absence of urocortin 2 (a CRH-R2 agonist), antisauvagine-30 or astressin; and D, soleus muscle indicating that at the concentrations used, the CRH receptor antagonists (antisauvagine-30 and astressin) did not alter basal  $\text{MO}_2$ . All data are on  $\text{MO}_2$  means, with vertical bars representing SEs ( $n = 5-7$ ). Significant effects in sequential changes in  $\text{MO}_2$  were assessed by paired  $t$  test or by ANOVA with repeated measures followed by Scheffé's test for pair-wise comparisons; in the latter case, the values not sharing the same superscript letters ( $a, b, c$ ) are significantly different from each other ( $P < 0.05$ ).



stimulate muscle  $\text{MO}_2$  in the presence of astressin, in contrast to that observed in contralateral muscles treated only with CRH (or urocortin 2) ( $P < 0.001$ ). Taken together, these studies indicate that CRH can directly stimulate thermogenesis in skeletal muscle, and that the thermogenic effects of CRH are consequential to the activation of specific CRH receptors.

#### Requirement for lipid oxidation and *de novo* lipogenesis

We have previously proposed that substrate cycling between *de novo* lipogenesis and lipid oxidation, a futile cycle known to be involved in brown adipose tissue thermogenesis (24), also operate as a thermogenic effector in skeletal muscle (15) on the basis of previously reported calorimetric studies indicating that inhibition of key control points along pathways in this substrate cycle resulted in the blunting of the direct effect of leptin in stimulating thermogenesis in skeletal muscle (14). After our findings above that CRH also stimulates skeletal muscle thermogenesis with a similar muscle type specificity (soleus > EDL) and similar magnitude (20% increase in soleus muscle) as previously reported for leptin (13, 14), we test here whether CRH might also operate through the same molecular mechanisms of substrate cycling. To this end, we used the same concentrations of inhibitors that we previously used to implicate a potential role of this substrate cycle in the direct effect of leptin on muscle thermogenesis (14).

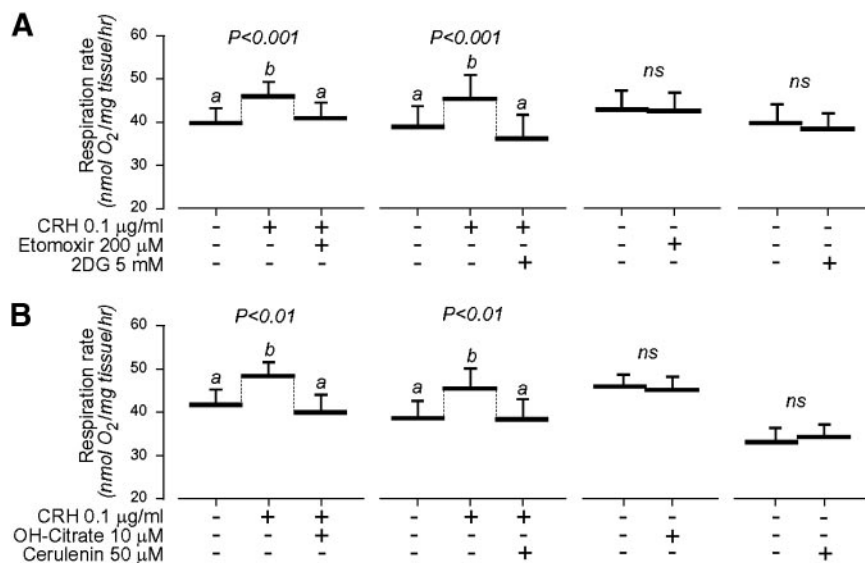
The results presented in Fig. 2A show that the direct effect of CRH on muscle  $\text{MO}_2$  is completely inhibited ( $P < 0.001$ ) by 1) etomoxir, a known selective inhibitor of carnitine palmitoyl transferase-1 (CPT-1), which regulates the entry of long chain fatty acids into mitochondria, and by 2) the replacement of glucose in the perfusion medium with 2-deoxyglucose, a glucose analog whose metabolism stops after its phosphorylation by hexokinase, thereby indicating that CRH-induced thermogenesis in muscle requires both fatty acid oxidation and glucose metabolism. A requirement of *de novo* lipogenesis for CRH-induced thermogenesis in skeletal muscle is suggested by data in Fig. 2B, showing that CRH-induced increases in muscle  $\text{MO}_2$  are completely abolished

( $P < 0.01$ ) by the addition of inhibitors of key control points in pathways leading to the synthesis of lipids, namely by either OH-citrate, which inhibits the enzyme citrate lyase, or by cerulenin, an inhibitor of fatty acid synthase. Taken together, these data suggest that lipid oxidation, glucose metabolism and *de novo* lipogenesis are all required for the direct thermogenic effect of CRH in skeletal muscle.

#### Requirements for PI3K and AMPK

We have also previously proposed that PI3K and AMPK are key signaling pathways required for the orchestration of this thermogenic substrate cycle between *de novo* lipogenesis and lipid oxidation in response to leptin (14, 15). To test whether PI3K and AMPK signaling are also involved in the direct thermogenic effects of CRH in skeletal muscle, we investigated here whether the increase in muscle  $\text{MO}_2$  in response to CRH might be abolished by inhibitors of PI3K or AMPK, at concentrations previously reported to be effective in blocking the thermogenic effect of leptin (13, 14). Furthermore, we also measured, using biochemical assays for PI3K and AMPK, whether these signaling pathways are stimulated in skeletal muscle incubated with CRH. The results, presented in Fig. 3A, show that the subsequent addition of either wortmannin or LY294002, two specific inhibitors of PI3K, completely abolished the CRH-induced increase in soleus  $\text{MO}_2$  ( $P < 0.001$  for wortmannin and  $P < 0.05$  for LY294002). Using *in vitro* kinase assays in *ex vivo* intact soleus muscles incubated with CRH, insulin, or saline solution as control, we could not, however, detect an induction of PY, IRS1, IRS2 and p85-associated PI3K activity, nor in Akt phosphorylation (a target of the PI3K pathway) with 0.1  $\mu\text{g}/\text{ml}$  CRH (Fig. 3, B and C) nor with 0.5  $\mu\text{g}/\text{ml}$  CRH (data not shown); this is in contrast to the expected marked increases in both PI3K and Akt signaling observed in response to insulin (Fig. 3, B and C). These data from biochemical assays, together with those obtained by calorimetry with the use of PI3K inhibitors, suggest that at least a basal PI3K activity is required (and possibly sufficient) for the thermogenic effect of CRH.

FIG. 2. Steady-state  $\text{MO}_2$  of soleus muscle (A) in the basal state, followed by sequential addition of CRH and etomoxir (an inhibitor of fatty acid oxidation), or during addition of CRH, and subsequently after addition of CRH in buffer in which glucose has been replaced by 2-deoxyglucose (2-DG), and (B)  $\text{MO}_2$  in the basal state, during sequential addition of CRH and OH-citrate, an inhibitor of citrate lyase, or during sequential addition of CRH and cerulenin, an inhibitor of fatty acid synthase. Note that at the concentrations used, the inhibitors did not alter basal  $\text{MO}_2$ . All data are means, with vertical bars representing SEs ( $n = 5-6$ ). Statistical significance of differences are shown as indicated in the legend to Fig. 1.



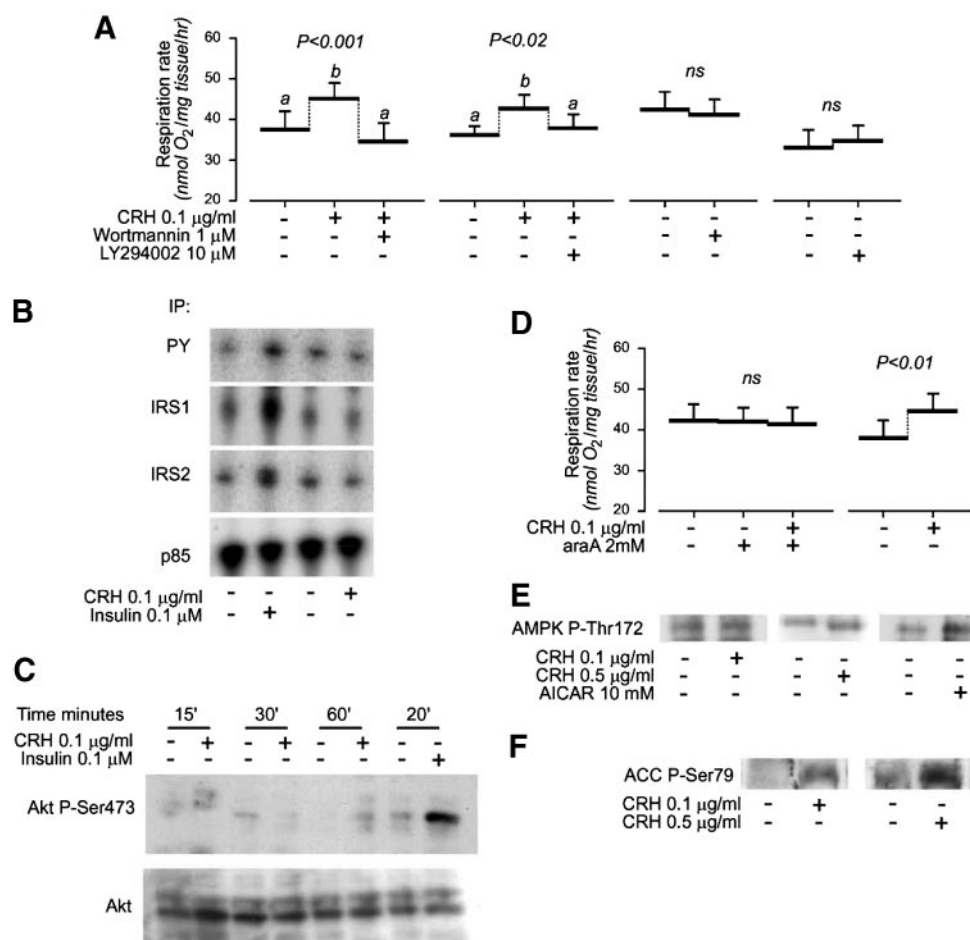


FIG. 3. A, Steady-state  $MO_2$  of soleus muscles in the basal state, during sequential addition of CRH and wortmannin or LY294002 (inhibitors of PI3K); note that at the concentrations used, these inhibitors have no effect on basal  $MO_2$ ; B, immunoprecipitate (IP) of PY, IRS1, IRS2, and p85 associated with PI3K activity from soleus muscles in response to CRH or to insulin as a positive control; C, Immunoblot showing Akt phosphorylation from soleus muscle in response to CRH or to insulin as a positive control; D, steady-state  $MO_2$  of soleus muscles in the basal state, during the sequential addition of araA (a competitive inhibitor of AMPK) and CRH or the response of contralateral muscles to CRH in absence of araA; and E, Western blot showing that AMPK phosphorylation is increased marginally in soleus muscles incubated with 0.1  $\mu$ g/ml CRH, but by about 2-fold in soleus muscles incubated with either 0.5  $\mu$ g/ml CRH or 10 mM of 5-aminoimidazole-4-carboxamide ribonucleoside (a well-known activator of AMPK) as a positive control; F, Western blots showing that ACC phosphorylation is also increased in response to CRH. For the calorimetry data, all data are means, with vertical bars representing SES ( $n = 5-6$ ); statistical significance of differences are shown as indicated in the legend to Fig. 1. For the PI3K, Akt, AMPK, and ACC assays, the effect of each hormone or drug was investigated by comparing pools of four left leg soleus muscles (hormone or drug treated) and four right leg soleus muscles (mock controls) from the same animals, and the experiment was replicated three times.

A requirement for AMPK signaling is also indicated by data presented in Fig. 3D showing that prior addition of adenine araA, an intracellular competitive inhibitor of AMPK, prevents CRH-induced increases in muscle  $MO_2$ , and that both AMPK and ACC phosphorylation are increased after treatment of skeletal muscle with CRH (Fig. 3, E and F), these increases being marginal with 0.1  $\mu$ g/ml CRH ( $<20\%$ ; not significant), but significant with 0.5  $\mu$ g/ml CRH ( $+40-120\%$ ,  $P < 0.01$ ). Taken together, these data suggest that both PI3K and AMPK activities are required for the direct action of CRH in stimulating skeletal muscle thermogenesis.

## Discussion

We show here, for the first time, that CRH can directly stimulate thermogenesis in skeletal muscle, an effect that is

much more pronounced in oxidative muscles (soleus) than in glycolytic muscles (EDL). This thermogenic response to CRH in skeletal muscle could be completely blocked by the non-selective CRH receptor antagonist astressin, thereby showing that this response is dependent upon the activation of specific CRH receptors. To date, only one CRH receptor isoform, CRH-R2 $\beta$ , has been described in skeletal muscle (19, 20). Our data showing that the thermogenic effect of CRH can be mimicked by urocortin 2 (a CRH-R agonist with high affinity for CRH-R2), and that it can be partially inhibited by antisauvagine-30 (a selective CRH-R2 antagonist), are consistent with a role for CRH-R2 in the peripheral effect of CRH on muscle thermogenesis. At present, we cannot rule out whether the partial inhibitory effects of antisauvagine-30 on thermogenic actions of CRH or that of urocortin 2 are due to a partial inhibition of CRH-R2 $\beta$  or because skeletal muscle

expresses other CRH receptor isoforms that are not yet identified. This latter explanation might assume greater importance after the observation, by immunohistochemistry analysis of both oxidative and glycolytic skeletal muscles, that the CRH-R2 $\beta$  protein could be localized in neural structures, blood vessels, myotendinous junctions, and endomysial/perimysial spaces, but not in myocytes (25). On the other hand, the report that CRH-R2 expression is detectable in primary cardiomyocytes cell culture by RT-PCR suggests that this receptor subtype is also expressed in muscle cells (26). Taken together, these observations raise questions as to whether the differential thermogenic responses to CRH observed here in mouse soleus and EDL muscles (which we found to show similar gene expressions levels of CRH-R2 $\beta$ ) would reside 1) in the abundance of this putative unidentified CRH receptor isoform in myocytes, 2) in the relative abundance of CRH-R2 $\beta$  in different tissues/structures within the muscle, or 3) in events downstream to CRH-R2 $\beta$  signaling. Furthermore, because the heart, like skeletal muscle, also expresses CRH-R2 $\beta$  and urocortin 2 (27), the questions arise as to whether the localization of CRH-R2 $\beta$  receptors in the heart muscle is fundamentally different from that in skeletal muscle, and whether urocortin 2—a potentially important ligand to the CRH-R2 $\beta$  in both muscles—plays an autocrine/paracrine role in controlling substrate metabolism and thermogenesis in these tissues.

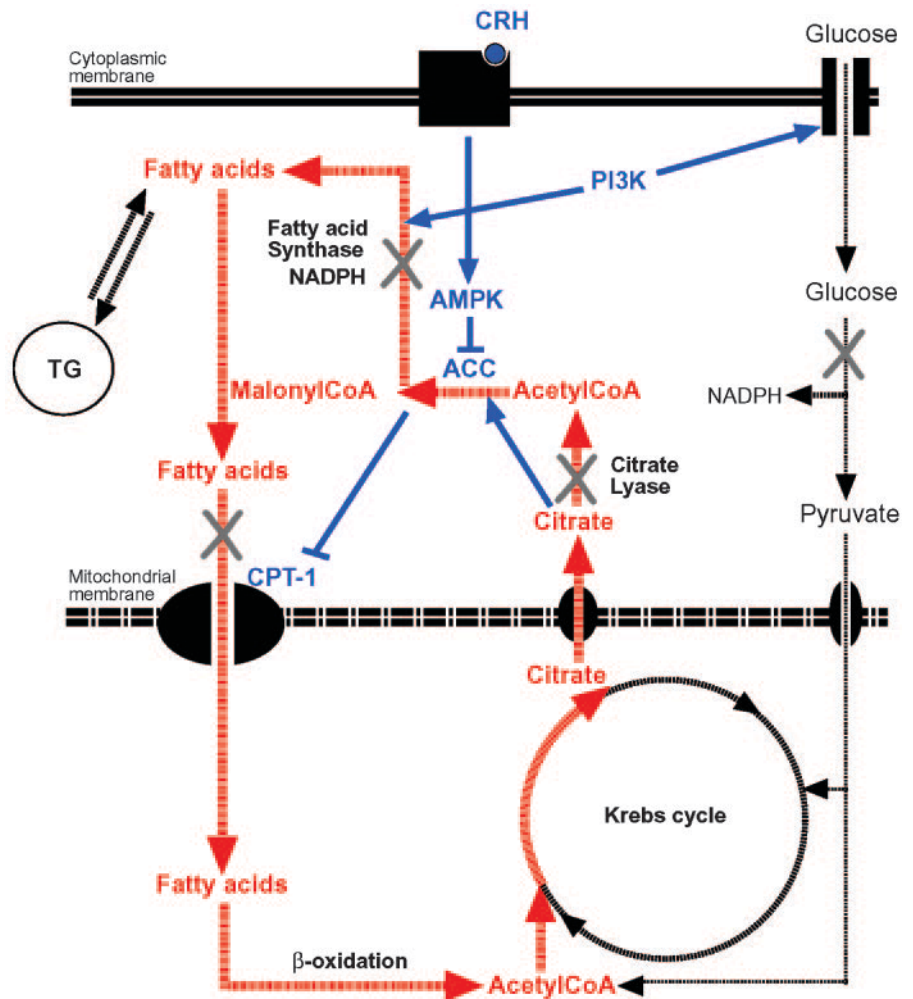
Despite considerable search over the past decades for effectors of thermogenesis in skeletal muscle, there is to date no convincing evidence that this tissue is a site of regulated mitochondrial uncoupling, by analogy to that mediated by the uncoupling protein (UCP1) in brown adipose tissue. In fact, the notion that the uncoupling protein homologs, UCP3 and UCP2, could be physiological regulators of thermogenic uncoupling in skeletal muscle has not received experimental support despite considerable testing (28). This point is further illustrated by the recent demonstration that constitutive UCP3 overexpression at physiological levels in mouse skeletal muscle, while increasing the capacity of this tissue for fatty acid oxidation, nonetheless failed to increase thermogenesis (29). Given doubts that regulated mitochondrial uncoupling underlies thermogenesis in intact muscle, there is nowadays renewed interest about the potential role of futile cycles toward understanding the biochemical basis of thermogenesis in general and in skeletal muscle thermogenesis in particular. In this context, an increased rate of substrate cycling between triacylglycerol and fatty acid has recently been shown to account for about 15% of the thermogenic effect of leptin administered *in vivo* (30), and we have previously reported that leptin can directly stimulate thermogenesis in oxidative muscle *ex vivo* (13) possibly by promoting an energy dissipating substrate cycling between *de novo* lipogenesis and lipid oxidation (14, 15)—a futile cycle that is also known to be involved in cold-induced thermogenesis in brown adipose tissue (15, 24). We present here data indicating that the direct effect of CRH on skeletal muscle thermogenesis is also dependent upon this flux of substrates between *de novo* lipogenesis and fatty acid oxidation because the increase in muscle O<sub>2</sub> consumption in response to CRH is completely inhibited by interference with either 1) the metabolism of glucose, using 2-deoxyglucose; 2) the conver-

sion of citrate to acetyl-CoA, using the citrate lyase inhibitor hydroxycitrate; 3) the conversion of malonyl-CoA to fatty acids, using cerulenin, an inhibitor of fatty-acid synthase; or with 4) the entry of fatty acids into mitochondrial  $\beta$ -oxidation pathway using etomoxir, an inhibitor of CPT-1. It is proposed that during CRH-induced activation of this substrate cycle (depicted in Fig. 4), acetyl-CoA produced from fatty acid and glucose oxidation, will overload the Krebs cycle. This will result in excess mitochondrial citrate that, in the cytoplasm, will exert an allosteric activation of the enzyme ACC and at the same time, under the action of citrate lyase, will provide acetyl-CoA to ACC for the synthesis of malonyl-CoA. The latter will serve as the main substrate for fatty acid synthase, thereby producing a new pool of fatty acids. Glucose plays a central role in this cycle as a source of Krebs cycle intermediates and reduced nicotinamide adenine dinucleotide phosphate molecules that are required for *de novo* synthesis of fatty acids (31). The capacity of skeletal muscle to perform *de novo* lipogenesis is strongly supported by the recent demonstrations in muscle satellite cells that glucose stimulates the expression of sterol regulatory element binding protein-1c mRNA as well as key genes encoding glycolytic and lipogenic enzymes, leading to an increased lipogenic flux and intracellular lipid accumulation (32, 33). These findings in primary muscle cell cultures provide direct evidence that *de novo* lipogenesis can occur in rat and human skeletal muscle cells and are consistent with data of metabolic labeling from our laboratory showing that *de novo* lipogenesis can also occur in intact mouse soleus and EDL muscles used in our calorimetric studies (14, 15).

Our data also suggest that this CRH-induced substrate cycling between *de novo* fatty acid synthesis and fatty acid oxidation in skeletal muscle is coordinated by AMPK and PI3K signaling because 1) the thermogenic effect of CRH in the soleus muscle, which is associated with AMPK and ACC phosphorylation, can be prevented by pharmacological inhibition of AMPK activation using araA, and because 2) the effects of CRH on muscle thermogenesis can also be completely inhibited by inhibitors of PI3K (wortmannin or LY294002). Because our data also indicate that the requirement for PI3K activity in CRH-mediated thermogenesis in skeletal muscle is independent of a CRH-mediated induction of PI3K activity, it is possible that basal levels of PI3K activity (inhibited by wortmannin or LY294002) are required to support the direct thermogenic effect of CRH in skeletal muscle. Further evidence underscoring a dual requirement for AMPK and PI3K signaling in the operation of this substrate cycling can be derived from our previous findings (14) that the administration of insulin (a potent stimulator of PI3K activity) to our muscle preparations is not sufficient to induce skeletal muscle thermogenesis, and that the activation of AMPK by 5-aminoimidazole-4-carboxamide ribonucleoside (or by leptin) led to an increase in muscle thermogenesis that is abolished by the PI3K inhibitor wortmannin (14). Thus, as depicted in Fig. 4, in response to CRH, AMPK-induced phosphorylation of ACC, will counterbalance the stimulatory action of citrate on ACC thereby resulting in reduced malonyl-CoA concentration, disinhibition of CPT-1, and increased fatty acid oxidation. This in turn will lead to the production



FIG. 4. Model illustrating an energy-dissipating futile substrate cycling between *de novo* lipogenesis and lipid oxidation in response to CRH, orchestrated by PI3K and AMPK signaling, in skeletal muscle (see text for details). TG, Triglycerides. The proposed model of substrate cycling in response to CRH rests upon data from calorimetric studies showing that the direct effect of CRH on skeletal muscle thermogenesis is inhibited by pharmacological interference at any one of key control points in the flux of substrates denoted by symbol (X), namely: 1) with glucose metabolism using 2-deoxyglucose (2-DG); 2) with the conversion of citrate to acetyl-CoA using hydroxy-citrate, an inhibitor of citrate lyase; 3) with the conversion of malonyl-CoA to fatty acids using cerulenin, an inhibitor of fatty acid synthase; and 4) with the entry of fatty acid into mitochondrial  $\beta$ -oxidation pathway using etomoxir, an inhibitor of CPT-1.



of acetyl-CoA that will fuel the Krebs cycle and increase citrate levels. Conversely, PI3K activity will promote cellular glucose uptake and synthesis of fatty acids. This would occur despite AMPK-induced reduction in malonyl-CoA because it is known that full phosphorylation of ACC by AMPK results in an inhibition of ACC activities only by 50–60% (34, 35). Such partial inhibition of ACC is expected to redirect the flux of acetyl-CoA and malonyl-CoA toward fatty acid oxidation but would still allow substantial rate of fatty acid synthesis, particularly in presence of high levels of citrate. According to the stoichiometry of this substrate cycle, the synthesis of one molecule of palmitic acid from acetyl-CoA and its reoxidation to acetyl-CoA would cost at least 14 molecules of ATP. Repeated recycling of acetyl-CoA through the flux of substrates across lipogenesis followed by  $\beta$ -oxidation could therefore constitute the mechanism by which CRH directly stimulates thermogenesis in skeletal muscle. The increases in thermogenesis observed here with CRH are quite similar to those observed with leptin, namely between 20 and 30% (13, 14), and higher than we observe with catecholamines (about 5–15% increase; Seydoux, J., and A. G. Dulloo, unpublished data). It should, however, be emphasized that because the skeletal mass is large (contributing between 30 and 40% of

total body mass and an even greater percentage of daily energy expenditure), a relatively small increase in muscle respiration rate can have a significant impact on whole body energy expenditure and on muscle lipid homeostasis. Consequently, the substrate cycling proposed here in response to CRH could also underscore a thermogenic effector system through which mobilization of the intramuscular triglycerides stores might have contributed to the increase in whole-body energy expenditure and fat oxidation observed in humans infused with CRH (21).

In conclusion, this study demonstrates that CRH can directly stimulate thermogenesis in skeletal muscle and provides evidence that raises the possibility that the direct action of CRH might be mediated by substrate cycling between *de novo* lipogenesis and lipid oxidation, orchestrated by PI3K and AMPK signaling. The capacity of CRH to directly stimulate metabolic rate by an energy dissipating substrate cycle linking glucose and lipid metabolism to thermogenesis in skeletal muscle provide the rationale for the development of peripheral CRH receptor agonists as potential thermogenic drugs in the management of obesity or against excessive accumulation of intramyocellular lipids, and hence against skeletal muscle lipotoxicity and insulin resistance.

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